Identification and Characterization of a *Salmonella* Hyper-biofilm isolate from mouse gallstones

Undergraduate Honors Thesis

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Chapter 1

Introduction

1.1. Problem Statement

Typhoid fever, a human-specific disease, affects approximately 21 million people each year, resulting in about 200,000 deaths[1]. The causative agent of typhoid fever is *Salmonella enterica* subsp. *Enterica* serovar Typhi (*S*. Typhi). Typhoid mainly affects underdeveloped areas of South Central Asia, Southeast Asia, Latin America and Southern Africa[2]. The disease is commonly spread by food or water contaminated through bacterial shedding in feces and urine of infected people or carriers of *S*. Typhi[3]. Once ingested, the bacteria cross the intestinal epithelial barrier, are phagocytosed by macrophages, and spread throughout the body in the bloodstream, often infecting the ileum, liver, spleen, bone marrow, and gallbladder[5]. It is estimated that 3-5% of people affected with typhoid fever become chronic carriers, and of those, 25% experience no clinical symptoms during the acute phase of the disease, resulting in greater spread of the organism[4,5]. Carriage in humans occurs primarily in the gallbladder.

The infection of the gallbladder is of special interest to this work. Studies have shown a correlation between the typhoid fever carrier state and the presence of gallstones, with 90% of carriers presenting with gallstones[2]. This correlation has been shown to be the result of the formation of *Salmonella* gallstone surface biofilms, an aggregation of bacteria encased in an extracellular matrix, adhering to each other and the gallstone surface[6] (Figure 1A). Biofilm formation allows the organism to withstand inhospitable environments, including the bile-filled gallbladder, resulting in persistence within the host. Bacteria are occasionally shed from the biofilm into the feces and urine. Because typhoid fever is a human-specific disease, the carrier state plays a crucial role in its spread. Currently, the exact mechanism of biofilm formation is

not completely understood and the only effective treatment is gallbladder removal, which is rarely a viable option in countries where typhoid fever is prevalent. Overall, the goal of this project was to gain a greater understanding of the mechanisms which result in this carrier state, specifically focusing on potential changes to *Salmonella* caused by host-pathogen interactions. By better understanding these mechanisms, it may be possible to develop new non-surgical techniques to resolve the chronic carrier state, thereby alleviating the spread of typhoid fever.

1.2. Identification of a Hyper-biofilm Mutant

In order to study the pathogenesis of *S*. Typhi, a human-specific pathogen, *in vivo*, *Salmonella enterica* subsp. *Enterica* serovar Typhimurium (*S*. Typhimurium) is used in a mouse model as *S*. Typhimurium causes a typhoid fever-like disease in mice[7]. In the course of a previous long term study of gallstone-bearing mice infected with *S*. Typhimurium, a *S*. Typhimurium isolate was recovered from a biofilm on a mouse gallstone 9 months post-infection. This isolate exhibited increased biofilm formation over the wild type strain, a phenotype which persisted when the strain was passaged repeatedly (Figure 1B). The persistence of this hyper-biofilm phenotype suggested that it was due to a genetic mutation and not a transient change in gene expression. The discovery of this mutant strain may represent a random occurrence or it may be a directed change within the bacterium in response to the host environment to facilitate biofilm formation, thereby enhancing colonization and persistence in the host gallbladder. *Pseudomonas aeruginosa*, a pathogen of the cystic fibrosis lung, directs a similar genetic change in which it becomes mucoid, fostering a biofilm phenotype and lung persistence[8]. Similarly, *Salmonella* is known to undergo an *in vivo*-induced alteration of the lipopolysaccharide (LPS) component of its outer membrane that aids its ability to avoid detection

by the immune system[9]. Given this precedent, it is possible that another such mechanism exists which results in the observed hyper-biofilm phenotype. The purpose of the previous long-term growth experiment was to determine the long-term health effects of the carrier state, with the isolation of the hyper-biofilm mutant as an incidental finding. Because the hyper-biofilm state has not been the focus of any study, no data is available on the prevalence of this phenotype.

1.3. Hypothesis and Rationale

The central hypothesis for this thesis is that within the gallbladder, *Salmonella* undergoes a controlled genetic modification in response to the local microenvironment that enhances gallstone biofilms and persistent gallbladder colonization. The recovered hyper-biofilm isolate formed the cornerstone of this hypothesis and thus the study of three major aspects of this isolate were examined:

1. Identification of mutations in genes responsible for the hyper-biofilm state.
2. Examination of biofilm and virulence-related properties of the hyper-biofilm strain.
3. Relationship of the identified gene mutations to the mechanism of hyper-biofilm formation.

The hyper-biofilm isolate we recovered grants us a unique insight into the *in vivo* process of biofilm formation on cholesterol gallstones. *Salmonella* species are known to respond to the *in vivo* environment to alter pathogenic properties and avoid host immune clearance. Previous studies have also shown *P. aeruginosa*-controlled genetic mechanisms that affect biofilm formation in the cystic fibrosis lung. Given these previous findings, we believe it is reasonable to suggest that a controlled genetic modification is responsible for the creation of our hyper-

biofilm isolate as well. The three specific aims listed above allow us to characterize our mutant in relation to wild type *Salmonella* and study its capacity for this genetic switch *in vivo*.

Chapter Two

Materials and Methods

2.1. Bacterial Growth Conditions

Wild type *S.* Typhimurium ATCC 14028S and its derivatives were used in this study, with specific strains listed in Table 2.1. Cultures were grown in Luria-Bertani (LB) broth, occasionally supplemented with 3% ox bile (Sigma-Aldrich, St. Louis, MO) for bacterial growth, biofilm assays, and creation of mutants, unless otherwise stated. When necessary, antibiotics were used in the following concentrations: ampicillin, 100 µg/ml; kanamycin, 45 µg/ml; chloramphenicol, 25 µg/ml.

For long-term growth experimentation, cultures were grown in LB broth supplemented with 3% ox bile (Sigma-Aldrich, St. Louis, MO), with or without the presence of a human gallstone for sixty days, with media replaced daily. Gallstones were primarily composed of cholesterol and were obtained from Dr. Wayne Schwesinger at the Univ. of Texas HSC at San Antonio. These samples were determined to be exempt from IRB approval.

2.2. Biofilm Growth on Microtiter Plates

Glass bottom 12-well plates (14-mm-diameter microwells; glass; no 1.5; MatTek Corp., MA) uncoated or coated by evaporation with 4 mg of cholesterol (diluted in ether; anhydrous; J. T. Baker, NJ) were inoculated with 2×10^8 bacteria in 2 ml of LB with or without 3% ox bile (Sigma-Aldrich, St. Louis, MO). The plates were incubated for 24 hours at 37°C in a GyroMini nutating mixer at 24 rpm (LabNet International, Inc., NJ).

Isolates from the long-term growth experiment were tested in plastic bottom 24-well plates (Thomas Scientific, NJ). Overnight cultures were grown in LB broth and normalized to an

optical density at 600 nm (OD_{600}) of 0.8, and diluted 1:100 in TSB (diluted 1:20), and grown at 30°C under static conditions.

2.3. Crystal Violet Assays

Biofilms attached to the microtiter walls or cholesterol-coated coverslips were washed with 1x phosphate-buffered saline (PBS) and heat fixed at 60°C for one hour. Following fixation, the biofilms were stained with 0.25% crystal violet for five minutes. Microtiter wells were then washed three times with 1x PBS, after which a 33% acetic acid solution was used to extract the dye. Crystal violet retention was measured via use of a SpectraMax Spectrophotometer with SoftMaxPro software at an optical density of 570 nm (OD_{570}). Biofilms for each strain were grown in triplicate to ensure consistency of results. To compensate for background absorbance, OD_{570} values for noninoculated cholesterol-coated coverslips were averaged and subtracted.

2.4. Biofilm Disruption Assays

Biofilms of various *S.* Typhimurium strains were initially grown for 24 hours as listed above. Following this incubation period, media was removed from each well and replaced with new LB supplemented with one of several enzymes chosen to test for the presence of various potential biofilm components. These enzymes included DNAse (56 units/well), proteinase K (14 µL/well), and cellulase (50 units/well). The plates were then incubated for 20 hours at 37°C in a GyroMini nutating mixer (LabNet International, Inc., NJ).

2.5. Motility Assay

Overnight cultures of *S.* Typhimruium strains were diluted 1:100 and incubated on a rotating drum at 37°C to reach mid-to-late exponential phase growth [optical density at 600 nm (OD_{600}) 0.6-0.8]. Cultures were then normalized and 3 µl of each culture was inoculated into the center of an LB plate containing 0.3% agar. These plates were then incubated at 37°C for 24 hours, after which the radius of growth was measured on each plate.

2.6. Cellulose Production Assay

Cellulose production was assessed via a modified broth calcofluor-binding assay. Overnight cultures were grown in LB broth without salt (LBNS) and normalized by optical density. These cultures were then diluted 1:100 in LBNS broth containing calcofluor (20 µg/ml; Sigma) in black 96-well microtiter plates (Corning Costar, Cambridge, MA) and incubated in the dark at 22°C for 6 days[10]. The fluorescence (excitation = 366 nm, emission = 565nm) of each well was measured using a Spectra Max M2 Plate reader.

2.7. Electron Microscopy

For Scanning Electron Microscope (SEM) observations, biofilms on glass or cholesterol were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer-0.1 M sucrose (pH 7.4), rinsed twice with 0.1 M phosphate buffer, and dehydrated by the addition of solutions of ethanol in a graded series as follows: 35%, 50%, 70%, 80%, 95%, and 100%. Samples were chemically dried with consecutive washes of 25%, 50%, 75%, and 100% hexamethyldisilazane (Ted Pella, CA). Samples were dried overnight in a fume hood, mounted on aluminum stubs, and

sputter coated with gold for observation using an FEI Nova NanoSEM at the OSU Campus Microscopy and Imaging Facility (CMIF).

For Transmission Electron Microscope (TEM) observations, overnight cultures of *S.* Typhimurium strains were rinsed with 1x PBS and resuspended in 200 µl of 1.5% paraformaldehyde–1.5% glutaraldehyde in 0.1 M phosphate buffer to fix the cells. Cultures were stained using a 2% uranyl acetate and visualized using an FEI Technai G2 Spirit transmission electron microscope (CMIF, The Ohio State University).

2.8. Western Blotting

Monoclonal antibodies against *Salmonella* species flagella (Maine Biotechnology Services, Portland, ME) were used to detect flagellin in whole cell lysates from *S.* Typhimurium wild type, various hyper-biofilm strains, and a *fliC/fljB* mutant. Overnight cultures were grown in LB broth and normalized to OD_{600} 1.5. Cells were pelleted and the supernatant was removed. The pellets were resuspended in 75 µL of Lamelli loading buffer. Samples were boiled for 15 min and 15 µl of each sample was loaded onto a 15% SDS-PAGE for separation. Proteins were transferred for 1h at 60 V to MeOH-activated PVDF membrane and blocked overnight in 5% BSA in TBS. The membrane was washed in TBST (3x10 min), incubated with anti-flagella antibody (1:10,000 in 5% BSA/TBST for 2 h, 22°C) washed in TBST (3x10 min) and incubated with HRP conjugated goat α-mouse (1:12,000 in 5% BSA/TBST for 2 h, 22°C). The membrane was once again washed in TBST (3x10min) and visualized using the Bio-Rad ChemiDoc System.

Polyclonal antisera against *E. coli* CsgA (gift of Dr. Matthew Chapman, University of Michigan) were used to detect curli fimbriae in whole cell lysates from *S.* Typhimurium wild

type, various hyper-biofilm strains, and a *CsgA* deletion mutant. This western was conducted as previously described.[11] The bacterial strains were grown on LBNS agar for 6 days, after which cells were scraped from plates, resuspended in 1 mL 1xPBS pH 7.4 and normalized to OD_{600} 0.8. Cells were pelleted again and resuspended in 80 µL of 88% formic acid to depolymerize CsgA. Samples were dried via Speed-vac and resuspended in 40 µL H_2O. Lamelli loading buffer was added to a final volume of 80 µL and 10 µL was loaded onto a 10% SDS-PAGE for separation. Proteins were transferred for 1h at 60 V to MeOH-activated PVDF membrane and blocked overnight in 5% BSA in TBS. The membrane was washed in TBST (3x10 min), incubated with anti-CsgA antisera (1:10,000 in 5% BSA/TBST for 2 h, 22°C) washed in TBST (3x10 min) and incubated with HRP conjugated goat α-rabbit (1:12,000 in 5% BSA/TBST for 2 h, 22°C). The membrane was once again washed in TBST (3x10min) and visualized using the Bio-Rad ChemiDoc System.

2.9. Generation of Mutants and Cloning Procedures

Mutations of various genes of interest were performed by using the λ-red mutagenesis method[12]. To create a complemented strain of the deletion mutants, the wild type genes were cloned in pWSK29. In order to mimic the mutations observed from sequencing various hyper-biofilm strains, the wild type genes were altered via site-directed mutagenesis with a kit (Thermo Fisher Scientific Inc., Waltham, MA) to match the observed single nucleotide polymorphisms (SNPs). Oligonucleotide primers used to perform gene deletion, cloning, and site-directed mutagenesis are listed in Table 2.

2.10. Mouse Infections

In order to study the typhoid fever carrier state, a mouse model of infection using *S.* Typhimurium was employed. Naturally resistant *Nramp1*$^{+/+}$ 129X1/SvJ mice (Jackson Laboratory, ME) were fed a litogenic diet (1% cholesterol and 0.5% cholic acid; Sigma) or normal chow (Harlan Laboratory, IN). After six weeks, mice were infected intraperitoneally with 10^4 *S.* Typhimurium bacteria and sacrificed at 8 and 22 days postinfection (p.i.). Liver, gallbladder, bile, and gallstone (where appropriate) were homogenized and/or diluted for bacterial enumeration using LB agar.

2.11. Sequencing

Two rounds of sequencing were performed with various collaborators to identify mutations in the hyper-biofilm strain. The first round was performed by Dr. Wolfgang Sadee at the Ohio State University Program in Pharmacogenetics. The sequences were analyzed using Ion Torrent software and by CLCBio and compared to a reference strain (*Salmonella enterica* subsp. *enterica* serovar Typhimurium 14028S), a previously sequenced, complete genome for S. Typhimurium in order to identify SNPs. The second round of sequencing was performed by Dr. Peter White at the Nationwide Children's Hospital Biomedical Genomics Core. For this round, the sequencing was performed using Ilumina MiSeq technology to compare sequences from a larger set of isolates.

Chapter 3

Results

3.1. Gene Identification

3.1.1. Sequencing

The first step in identifying the gene or genes responsible for the formation of the hyper-biofilm state was whole genome sequence of both our wild type *S. Typhimurium* as well as the hyper-biofilm isolate recovered from the mouse model. The genomes were mapped as compared to a reference strain (*S.*Typhimurium 14028S) in order to generate a list of single nucleotide polymorphisms (SNPs). Using this list, we eliminated any mutations also found in our wild type as well as any silent mutations, resulting in 14 gene mutations which may be responsible for the hyper-biofilm phenotype. These mutations were then ranked according to the likelihood of their effect on biofilm growth (Table 3). As this number was too high to effectively test all candidates for links to a hyper-biofilm phenotype, a second round of sequencing with additional strains was performed to further narrow the list. A collection of *S.*Typhimurium isolates were recovered from the mouse gallstone sample that originally yielded the hyper-biofilm mutant. Testing of these additional isolates revealed that although all displayed enhanced biofilm formation several had differing motility phenotypes (Figures 2A and 2B). Additionally, isolates recovered from the bile of the same mouse did not show enhanced biofilm formation, despite their proximity to the gallstones. In a second round of sequencing, two isolates with differing motility were compared, along with the isolate from the bile. Genes listed in bold on Table 3 were found to be mutated in both hyper-biofilm isolates, regardless of motility phenotype, but not mutated in the bile isolate. This indicated that these genes were the most likely to be involved in biofilm

formation and not simply survival in the harsh bile environment of the gallbladder. This additional sequencing narrowed the list to four high priority candidates: *rcsB* and *envZ*, and two hypothetical proteins (STM14_1478, STM14_1618).

At the top of the list, we placed mutations to the *rcsB* and *envZ* genes. The *rcs* family of genes has been shown to be involved in colonic acid synthesis, cell wall formation, cell division, and most importantly for this project, biofilm development[13]. Thus, a mutation in the *rcsB* gene, which serves primarily as a regulator for the function of the Rcs family, could have a significant impact on biofilm development and morphology. EnvZ is a sensor protein in a two-component system with OmpR, which directs the cell's response to changes in osmolarity[14]. OmpR has also been connected to several signaling pathways involving biofilm development, and OmpR/EnvZ affect the synthesis of the Vi-antigen in *S*. Typhi, an important biofilm matrix component and a surface component that aids in the avoidance of immune system killing[15]. Given these factors, a mutation in the *envZ* gene could cause a major change in biofilm production.

Following the two-component system genes, two hypothetical proteins may be responsible for the hyper-biofilm state. Very little is known about the function of these genes, but their identification in the second round of sequencing gives a strong indication of their potential link to the hyper-biofilm state.

Proceeding down the original priority list are genes related to flagella formation. Flagella have been shown to play a key role in the initial surface attachment of *Salmonella* to cholesterol[9]. Given that surface attachment is the first stage of biofilm development, mutations in flagellar genes could have a large effect on *Salmonella*'s affinity for the cholesterol surface of the gallstone, thereby altering biofilm formation. The other genes on our list have little to no

known connection to biofilm formation, and as such no further experimentation with these genes has occurred at this time.

3.1.2. Generation of Mutants and Biofilm Testing

Several different techniques were used in order to create mutants with increasing resemblance to those discovered in the above sequencing. Beginning with the two highest priority candidates, the first step in this process was the deletion of the gene of interest. Genes were deleted using the λ-red recombinase system developed by Wanner and Datsenko[12]. The *envZ and rcsB* deletion mutants were then tested to determine their biofilm forming capability with the results shown in Figure 3A. Although this method is a somewhat crude representation of the observed mutations, it was used to establish the relative importance of the genes to biofilm formation in general. The assay showed no significant alteration to biofilm formation following the deletion of *envZ*, *rcsB*, or in a double mutant lacking both genes (Figure 3A). Due to the possibility that the observed mutations result in an alteration of protein function as opposed to a complete loss of function (as would be seen in the deletion mutant), further mutagenesis work was performed. Wild type copies of *envZ and rcsB* were inserted into plasmid pWSK29, a low copy-number plasmid, to limit the effect of gene overexpression and cloned into their respective deletion strains. Site-directed mutagenesis was then performed on the plasmid-borne copy of the gene, replicating the exact SNPs observed from sequencing the hyper-biofilm isolate. Biofilm assays were then performed using these mutants complementing the chromosomal deletion, including empty vector controls to ensure overexpression of the target genes did not alter the biofilm phenotype. The results of these assays are shown in Figures 3B and 3C. None of the created mutants displayed significant alterations to biofilm formation as compared to their

deletion strains. A similar protocol has been planned but not yet performed for several hypothetical protein candidates.

3.2 Biofilm and Virulence Properties

3.2.1. Biofilm Composition

The first goal in characterizing the biofilm formed by our mouse isolate was determining whether it formed a biofilm with characteristics phenotypically similar to the wild type strain, or if the mutations caused a change in the basic biofilm phenotype. To answer this question, biofilms from wild type *S.* Typhimurium and the hyper-biofilm mutant were visualized via Scanning Electron Microscopy (SEM) on a variety of surfaces in order to compare morphologies. This experiment provided us with two interesting results. First, the hyper-biofilm mutant appeared to form a much thicker, more robust biofilm with a significantly different morphology on a cholesterol surface, chosen due to its similarity to gallstone composition (Figure 4A). Second, our hyper-biofilm isolate showed little to no biofilm formation on a glass surface, indicating that the mutant biofilm-forming ability is surface-dependent, with increased biofilm formation occurring only on gallstone-like surfaces (Figure 4A). This experiment suggested that the hyper-biofilm mutant forms a phenotypically different biofilm than the wild type.

Further experiments focused on determining the exact phenotypic differences in biofilm composition. Two experiments were performed to study cellulose, a major extracellular component of *Salmonella* biofilms[16]. Both the wild type and the hyper-biofilm mutant were streaked on Congo Red plates, on which colony morphology can be used determine the presence

or absence of cellulose and curli fimbriae, a cellular appendage linked to biofilm formation[15]. Both strains exhibited a red, dry, and rough phenotype, indicating the presence of both cellulose and curli fimbriae, though this experiment does not quantitatively show the presence of these substances (Figure 4B). To quantitatively measure cellulose production, we used a calcofluor experiment which fluorescently labels cellulose, allowing its production to be measured. This experiment showed no significant difference in cellulose production between the wild type and our mutant, indicating that cellulose production is not the cause of the phenotypic biofilm change (Figure 4B).

To more closely observe the compositions of the wild type and mutant biofilms, a series of biofilm disruption assays were performed. In this experiment, the biofilms for both strains were allowed to grow normally for 24 hours, after which specific enzymes were added to target major components of the biofilms. Three different enzymes were used in this experiment: DNAse, proteinase, and cellulase, to target DNA, protein, and cellulose, respectively, with results shown in Figure 3C. The experiment showed that DNAse had a larger effect on the hyper-biofilm isolate than the wild type (though this difference was not statistically significant), proteinase had a large effect on both biofilms, and cellulase had a negligible effect on both biofilms (Figure 4C).

3.2.2. Cellular Appendages

Because of the flagellar mutations found in our hyper-biofilm strain and the importance of cellular appendages in surface attachment, a phenotypic difference in flagellar appendages could be a significant cause of the change in biofilm phenotype. Previous experiments had demonstrated that all tested hyper-biofilm isolates displayed a deficiency in motility, though the

severity of this deficiency varied (Figures 2A and 2B). Cellular appendages of wild type *S.* Typhimurium were visualized and compared to those of hyper-biofilm isolates with different motility phenotypes. Using Transmission Electron Microscopy (TEM), we observed long, smooth, and thick flagella present on wild type *Salmonella* with significant but different alterations occurring to the hyper-biofilm isolates (Figure 5A). While the exact morphology of the hyper-biofilm isolates differed, both in general exhibited thinner and more numerous appendages than the wild type. These changes in surface appendage phenotype may be due to a down-regulation of flagella in the mutants and a subsequent up-regulation of fimbriae, leading to altered surface attachment. Western blots were performed using whole-cell lysates of the wild type and hyper-biofilm mutants to address this issue, comparing expression of two common flagellar proteins, FljB and FliC (Figure 5B). Both the wild type and low motility hyper-biofilm isolate displayed similarly high levels of FljB expression, and low levels of FliC expression, although the hyper-biofilm isolate expressed slightly more FliC. The relatively high motility isolate, however displayed a complete inversion of this phenotype, low expression of FljB and high expression of FliC. Total expression of flagellar protein between the three isolates appeared fairly stable.

To test for a change in fimbriae expression, additional western blots were performed against CsgA, the monomeric subunit of curli fimbriae. The western blots were also performed using whole-cell lysates of the wild type and hyper-biofilm mutants. Several difficulties have thus far prevented a successful western blot against CsgA. The membranes display high levels of background and non-specific binding. Additionally, a band of the proper size appears in a mutant strain of *S.* Typhimurium confirmed to be lacking the *csgA* gene. Therefore a proper western blot comparing expression of curli fimbriae has not been performed at this time.

3.2.3. Virulence

Given the key role biofilm formation plays in the carrier state of typhoid fever, the larger, more robust biofilm formed by the hyper-biofilm strain is hypothesized to result in increased persistence within the host. In order to evaluate the role of hyper-biofilm production in virulence and colonization abilities, an in vitro biofilm competition assay was first performed. The protocol for this experiment generally followed a typical biofilm assay, except some wells were inoculated with both wild type and hyper-biofilm strains. After 24 hours, bacteria were recovered from the wells for CFU enumeration in place of the usual crystal violet staining. The wild type strain used in this experiment contained a plasmid encoding an antibiotic resistance cassette to allow for enumeration of each strain individually. A ratio of 1.04:1 (wild type: hyperbiofilm) CFU $\log_{10}$ recovery from the wells indicated that that the wild type and hyper-biofilm strains equally colonized the cholesterol surface.

To compare the virulence of the strains, *Nramp1*$^{+/+}$ 129X1/SvJ mice were infected with wild type, or hyper-biofilm *S.* Typhimurium. Prior to inoculation, some of the groups of mice had been fed a cholesterol rich diet, thereby promoting the formation of gallstones. Additionally, the *Nramp1*$^{+/+}$ status of the mice helps them to avoid succumbing to the initial stages of the infection, instead favoring the carrier state, making the mice an ideal model for typhoid carriage. During the first round of experimentation, mice were sacrificed 8 and 22 days post infection, after which samples were taken from the liver, gall bladder, bile, and gallstones (when applicable), homogenized, and enumerated. Bacterial counts from the various organs of the hyper-biofilm strain were virtually undetectable, indicating a severe systemic virulence defect (Day 8 data shown in Figure 6B, Day 22 data not shown). Upon further evaluation, we

hypothesized this defect occurred due to an ability to escape the host macrophages, thereby preventing systemic virulence. Previous studies have shown that the protein EnvZ, whose gene is truncated in the hyper-biofilm strain, plays a key role in intra-macrophage survival via the formation of a vacuole and killing of the macrophage[17]. Because we had previously demonstrated that EnvZ does not alter biofilm formation, a second experiment was performed, augmenting the hyper-biofilm strain with the addition of a wild type *envZ* on a low copy plasmid to test the virulence of the hyper-biofilm state alone. Though this strain was able to invade the host gall bladder, recovery was still inconsistent and below the values seen in the wild type (Figure 6C). Additionally, the hyper-biofilm mutant was still absent from all liver samples, indicating that even with a functional copy of EnvZ there is still a systemic virulence defect.

3.3. Potential Mechanism of Hyper-Biofilm Formation

To determine if the formation of the recovered hyper-biofilm isolate occurred via a controlled genetic modification, or by chance alone, a long-term growth experiment was performed. Wild type *S.* Typhimurium was grown in LB media supplemented with 3% bile with or without the presence of a human gallstone. Media was replaced daily while the culture was allowed to grow for 60 days. After the 60 days, 100 individual bacterial colonies from bacteria recovered from gallstone biofilms or from cultures grown identically but without gallstones were tested for their biofilm forming ability. 27 isolates from the gallstones displayed hyper-biofilm properties, compared to 6 isolates from the media lacking gallstones. Additional genome sequencing of these mutants, looking for mutations that match those seen in the original hyper-biofilm strain, is planned but has not yet been performed.

Chapter 4

Discussion

In the course of a previous long-term study, a *S.* Typhimurium isolate which exhibited enhanced biofilm forming ability was recovered from a biofilm on a mouse gallstone. Due to the persistence of the hyper-biofilm phenotype after passaging, we can conclude that the change is the result of a mutation, not a transient change in gene expression. The purpose of this study is the further identification and characterization of the *Salmonella* isolate, in the hopes of gaining a greater understanding of the mechanisms that result in gallbladder colonization and establishment of the carrier state. We hypothesized that within the gall bladder, *Salmonella* undergoes a controlled genetic modification in response to the local microenvironment that enhances gallstone biofilms and persistent gallbladder colonization. This hypothesis was tested via three major approaches:

1. Identification of mutations in genes responsible for the hyper-biofilm state.
2. Examination of biofilm and virulence-related properties of the hyper-biofilm strain.
3. Relationship of the identified gene mutations to the mechanism of hyper-biofilm formation.

4.1. Gene Identification

Through full genome sequencing, we identified a list of fourteen genes with single nucleotide polymorphisms (SNPs) in the hyper-biofilm strain. These genes were ranked based on their currently known association with biofilm growth and development, as well as other criteria gathered from additional isolates of the original mouse gall bladder. This list includes two genes involved in two-component regulatory systems, two hypothetical proteins, several

flagellar proteins, and other genes of currently low priority. Mutations matching the observed mutations for *rcsB* and *envZ* were constructed as were deletions of the genes. All mutants showed no effect on biofilm production. As such, we can conclude that individually they do not play a crucial role in the creation of the hyper-biofilm phenotype or in biofilm formation in general. Previous studies have linked both of these genes to biofilm formation, although given the results shown here, it appears *Salmonella* has sufficient biofilm-forming compensatory mechanisms. An additional gene, the hypothetical protein STM14_1478 can likely be eliminated from consideration due to the presence of several other genes within the *Salmonella* genome which contain some of the same highly conserved regions, including STM14_1190 which is 97% identical. As a result, a mutation which affects the function of STM14_1478 could likely be compensated for by the remaining genes with conserved regions. At this time, the exact gene or genes responsible for the hyper-biofilm state have not been identified, though the most likely remaining candidate is STM14_1618, a hypothetical protein with no currently known function. The protein is not predicted to be secreted from the cell, but may have a short transmembrane domain. It has no known orthologs outside of the *Salmonella* genus. Due to their role in the initial attachment phase of *Salmonella* to a cholesterol surface, several flagellar proteins may also still be involved and are being investigated.

4.2 Biofilm and Virulence Properties

Numerous experiments have demonstrated phenotypic differences between wild type *Salmonella* and the hyper-biofilm isolate. Observed via SEM, the biofilms have drastic morphological differences on cholesterol-coated surfaces, while the hyper-biofilm mutant is completely unable to form biofilms on glass surfaces. These findings indicate that the enhanced

biofilm phenotype is surface dependent, favoring cholesterol-coated surfaces like those of gallstones, though the reason for this preference is not yet known. We can conclude, however, that the mutant is not simply forming more biofilm, as the biofilm architecture greatly varied. Additional experiments comparing the composition of the biofilm showed no differences in cellulose production, a slightly higher effect of DNAse on the hyper-biofilm strain, and similarly strong reactions to protease by both biofilms. These data may indicate that eDNA plays a more important role in hyper-biofilm morphology, though the differences were not statistically significant. There are, however, large differences in the expression of cellular appendages. TEM imaging showed vastly different cellular appendage phenotypes even between two hyper-biofilm isolates. Western blots confirmed relatively consistent amounts of total flagellar proteins between the strains, although the expression of the flagellar subunits was altered. An upregulation of curli fimbriae, a smaller cellular appendage linked to surface attachment[9] may be the most likely reason for the drastic biofilm phenotype, and could link back to helping explaining the hyper-biofilm strain. Further experimentation demonstrated that the hyper-biofilm strain does not out compete the wild type when colonizing a surface, as both were equally present. This could indicate that the hyper-biofilm strain is capable of integrating wild type *Salmonella* into its biofilm and vice versa, or may also conclude that its biofilm simply does not exclusively cover a surface, leaving room for other pathogens. Finally, the hyper-biofilm strain demonstrates a clear systemic virulence defect in the mouse typhoid fever model. Some of this defect is due to a nonsense mutation in *envZ*, encoding a protein crucial to survival and escape from the host phagosome. Even after this protein is restored, however, the virulence defect remained, indicating additional virulence factor defects.

4.3. Potential Mechanism of Hyper-Biofilm Formation

The source of the hyper-biofilm mutation provides another avenue of exploration. It is entirely possible that the mutation(s) responsible for the hyper-biofilm phenotype occurred completely by chance. If this is the case, study of the isolate can still lead to important information about the proteins and pathways important to the biofilm formation process in general. If, however, we are correct in hypothesizing that a controlled genetic mechanism resulted in the shift, the isolate could provide significant insights into the pathogenesis of typhoid fever. Following prolonged incubation in gallbladder-resembling conditions, 27 of 100 isolates recovered from gallstones (directed towards biofilm growth) displayed hyper-biofilm characteristics as opposed to 6 of the 100 isolates grown without gallstones (left to planktonic growth). This drastic difference appears to support our hypothesis of a genetic mechanism directing the shift to a hyper-biofilm phenotype in response to the host environment. The presence of some hyper-biofilm isolates in the group without gallstones suggests that bile may play a role in the process, but the significant increase in the gallstone samples further suggests that prolonged biofilm growth is key to the process.

4.4. Conclusions and Future Directions

Because typhoid fever is a human specific disease, carriers represent a crucial reservoir, occasionally shedding *Salmonella* into their feces and urine, contaminating food and water supplies, thereby perpetuating the spread of the disease. Biofilm formation within the gallbladder has previously been shown to be a key mechanism of persistence within the host. The presented experiments demonstrate that there may be a controlled genetic mechanism within the gallbladder environment, directing *Salmonella* toward a hyper-biofilm phenotype, thereby

increasing persistence and allow further spread of the disease. The genes, processes, and mechanisms crucial to this pathway are not yet identified, however. Sequencing of isolates recovered from the long-term growth experiments, could play a significant role in narrowing down the list of candidate genes, bringing us even closer to identifying the gene or genes responsible. These isolates may also help identify the processes that lead to the morphological changes in the *Salmonella* biofilm. Although the hyper-biofilm state is advantageous in persistence, *in vivo* experimentation revealed that it is extremely detrimental to systemic virulence. This defect may help to explain the rarity of the phenotype among clinical samples, as shed hyper-biofilm isolates will be defective in establishing infection in a new host. Given the results of the biofilm competition assay, we know that wild type and hyper-biofilm mutants are capable of co-colonization of cholesterol surfaces. This may provide an advantage to the *S.* Typhimruium over favoring a single phenotype. The hyper-biofilm can provide a stronger, more robust biofilm on the gallstone surface, increasing persistence while the wild type bacteria can be shed and more successfully contaminate food and water supplies.

In addition to sequencing isolates from the long-term growth experiment, several other experiments could strengthen the hypothesis. Following the sequencing, creation of mutants and biofilm assays can definitively prove the gene(s) responsible for the hyper-biofilm state. Once identified, clinical isolates could be sequenced as well, screening for matching mutations to estimate real-world prevalence. Shorter extended growth experiments could also be performed to try to narrow down the timeframe when the shift occurs. Performing RNA-seq at those times could allow for the identification of disregulation of genes and pathways responsible for the shift. This will ideally lead to ideas for new non-surgical techniques to resolve the carrier state, thereby mitigating the spread of typhoid fever.

Tables

Strain	Characteristic(s)	Source or reference
JSG148	ATCC 14208s, pagKD	ATCC
JSG210	ATCC 14208s (CDC6516-60); wild-type	ATCC
JSG1190	hin108::Tn10d-Cam fliC::Tn10 (FljB locked off; FljBFliC-); (14208s background)	Gift of B. Cookson
JSG3538	Hyper biofilm-forming isolate from mice gallstones after 9 months of JSG210 infection	This study
JSG3540	csgA::Kan (14028s background)	Gift of M. McClelland
JSG3687	envZ::Kan (14028s background)	Gift of M. McClelland
JSG3689	rcsB::Cam (14028s background)	Gift of C. Detweiler
JSG3690	envZ::Kan; rcsB::Cam (14028s background)	This study
JSG3700	Isolated from mouse bile 9 months post-infection (same mouse as JSG3538)	This study
JSG3743	ΔenvZ complemented with wild type envZ gene in pwsk29 plasmid (14028s background)	This study
JSG3744	ΔenvZ complemented with mutated envZ gene in pwsk29 plasmid - site directed mutation Trp to stop codon at 176th position (14028s background)	This study
JSG3745	empty vector control - pwsk29 transformed into 3687 (14028s background)	This study
JSG3746	ΔrcsB complemented with wild type rcsB gene in pwsk29 plasmid (14028s background)	This study
JSG3747	ΔrcsB complemented with mutated rcsB gene in pwsk29 plasmid (14028s background)	This study
JSG3748	empty vector control - pwsk29 transformed into 3689 (14028s background)	This study
JSG3752	Hyper biofilm-forming isolate from mice gallstones after 9 months of JSG210 infection - low motility (isolate 3)	This study
JSG3753	Hyper biofilm-forming isolate from mice gallstones after 9 months of JSG210 infection - partial motility (isolate 9)	This study

Table 1. Bacterial Strains and Relevant Characteristics

Name	Primer Sequence (5'-3')	Purpose
JG2621	AAAGCTTGCTGTAGCAAGGTAGCCCAATACATGAACGTGTAGGCTGGAGCTGCTTC	λ-Red deletion of *rcsB*
JG2622	CAGGCTGGGTAACATAAAAGCGATTTATTCTTTGTCCATATGAATATCCTCCTTAG	λ-Red deletion of *rcsB*
JG2631	GCTCTAGAGTAGCAAGGTAGCCCAATACATG	Cloning of *rcsB* in pWSK29
JG2632	CGGGATCCTTATTCTTTGTCTGTCGGACT	Cloning of *rcsB* in pWSK29
JG2633	GCTCTAGAGTACCGGACGGTTCTAAAGCATG	Cloning of *envZ* in pWSK29
JG2634	CGGGATCCTTATGCCTCTTTTGTCGTCCC	Cloning of *envZ* in pWSK29
JG2635	GGCGGTTACGGCGATGAGCGTCTGTCGCCAAAAGAG	Site-Directed Mutagenesis of *rcsB* (Lys149Glu)
JG2636	CTCTTTTGGCGACAGACGCTCATCGCCGTAACCGCC	Site-Directed Mutagenesis of *rcsB* (Lys149Glu)
JG2637	GGCGGCGCGTGACTGTTTATTCGTATACAGAATCGA	Site-Directed Mutagenesis of *envZ* (Trp176*)
JG2638	TCGATTCTGTATACGAATAAACAGTCACGCGCCGCC	Site-Directed Mutagenesis of *envZ* (Trp176*)
JG2678	GCGGGCTTTTTTATGGAGGCAATATGCCAGGTGTAGGCTGGAGCTGCTTC	λ-Red deletion of *STM14_1478*
JG2679	CGCTCATTTTAAAAGTCATTATGCCAGCCTCATATGAATATCCTCCTTAG	λ-Red deletion of *STM14_1478*
JG2680	TAACGATACGCGTGCCGGGTAGTGTGTTGGGTGTAGGCTGGAGCTGCTTC	λ-Red deletion of *STM14_1618*
JG2681	AGTGAGTTGTGTATCCATCTAGCCAACCATCATATGAATATCCTCCTTAG	λ-Red deletion of *STM14_1618*
JG2688	GCTCTAGAGCGGGCTTTTTTATGGAGGCAATATG	Cloning of *STM14_1478* in pWSK29
JG2689	CCCTCGAGTTATGCCAGCCTCACTATGTA	Cloning of *STM14_1478* in pWSK29
JG2690	GCTCTAGACGATACGCGTGCCGGGTAGTG	Cloning of *STM14_1618* in pWSK29
JG2691	CCCTCGAGCTAGCCAACCATTGCTAAATT	Cloning of *STM14_1618* in pWSK29
JG2696	TCATCATGCCGGACGAAGATAGCGATTTTCGTCTG	Site-Directed Mutagenesis of *STM14_1618*(Asn27Asp)
JG2697	CAGACGAAAATCGCTATCTTCGTCCGGCATGATGA	Site-Directed Mutagenesis of *STM14_1618*(Asn27Asp)

Table 2. Oligonucleotide primers used in the study

Function/Annotation	Genes
Two-component system	*rcsB, envZ**
Hypothetical proteins	**STM14_1478, STM14_1618**
Flagella	*fliB*, flgI, fliG, fliT*
DNA Rearrangements	STM14_0647 (integrase)
Carbohydrate metabolism	*malT, melR, ugpE,* STM14_0844
O-antigen synthesis	*rfbG*

Table 3. Mutations Identified by Sequencing

Fourteen SNPs were identified between the hyperbiofilm isolates, bile isolates, and wild type *S. Typhimurium.* The genes were sorted based on their known functions, unnamed genes are listed according to their position in the genome. Bolded genes were identified to have extra significance by a second round of sequencing. *Indicates that the amino acid change resulted in a termination of the amino acid sequence.

Figures

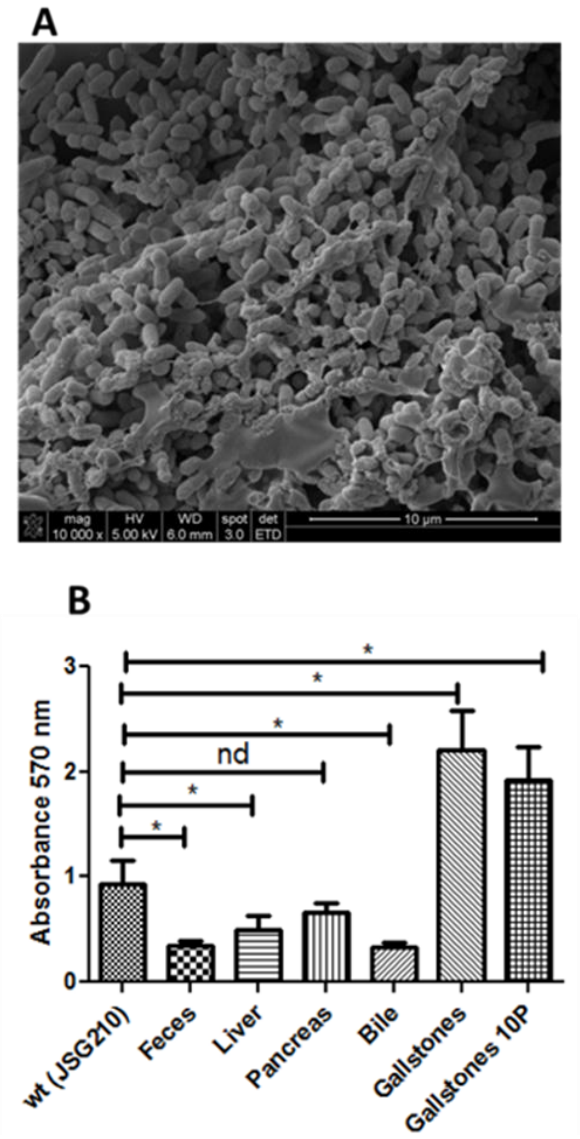


Figure 1. **Model of *Salmonella enterica* subsp. *enterica* serovar Typhi biofilm formation on cholesterol gallstones.**
A| Electron Micrograph of *Salmonella enterica* serovar Typhi in a biofilm on the surface of a human gallstone.
B| Biofilm capacity of isolates recovered from the different sites of a chronically infected mouse after 9 months of infection compared with the initial wild type inoculated strain (JSG210). The hyper-biofilm phenotype was specific to the gallstone environment (JSG3538) and was not lost after 10 laboratory passages. A Student's t-test was used to compare the wild type with each isolate (*$p<0.05$).

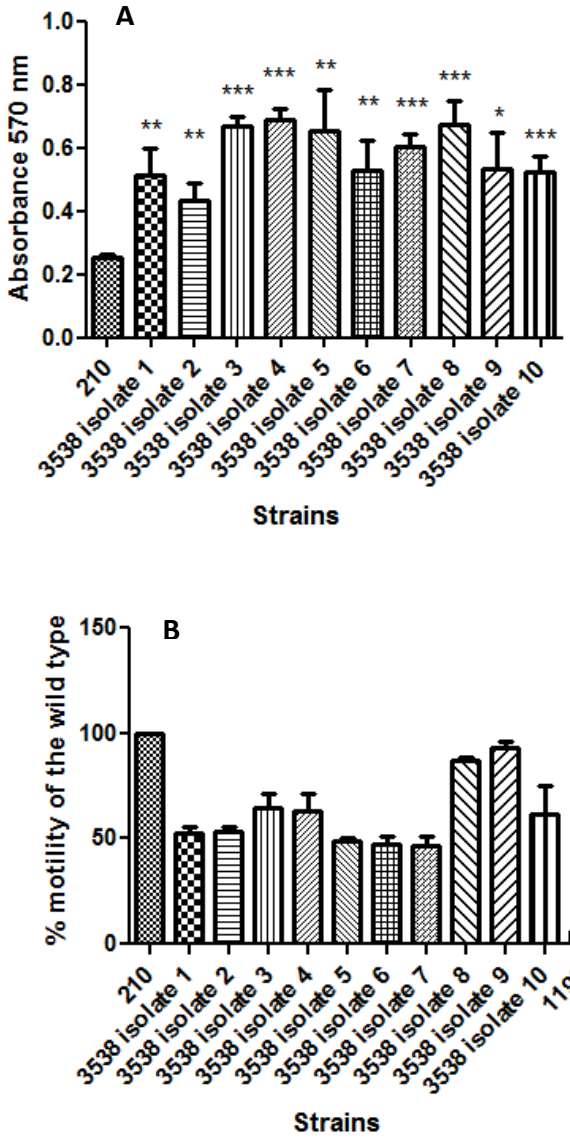


Figure 2. Biofilm Production and Motility of Mouse Gallstone Isolates

A| Biofilm production of isolates recovered from a mouse gallstone nine months post infection with *S.* Typhimurium. All isolates exhibited significant increase in biofilm production over the wild type. Statistical analysis confirmed using a Student‘s T-test (*p<.05; **p<.01; ***p<.001) **B|** Comparison of motility range for hyperbiofilm isolates as compared to the wild type. Eight of the ten isolates demonstrated large decreases in motility. The other two isolates displayed decreased motility from the wild type, but increased from the other hyperbiofilm mutants. Strain 1190 is a mutant lacking crucial flagellar proteins and incapable of motility, and was included as a negative control.

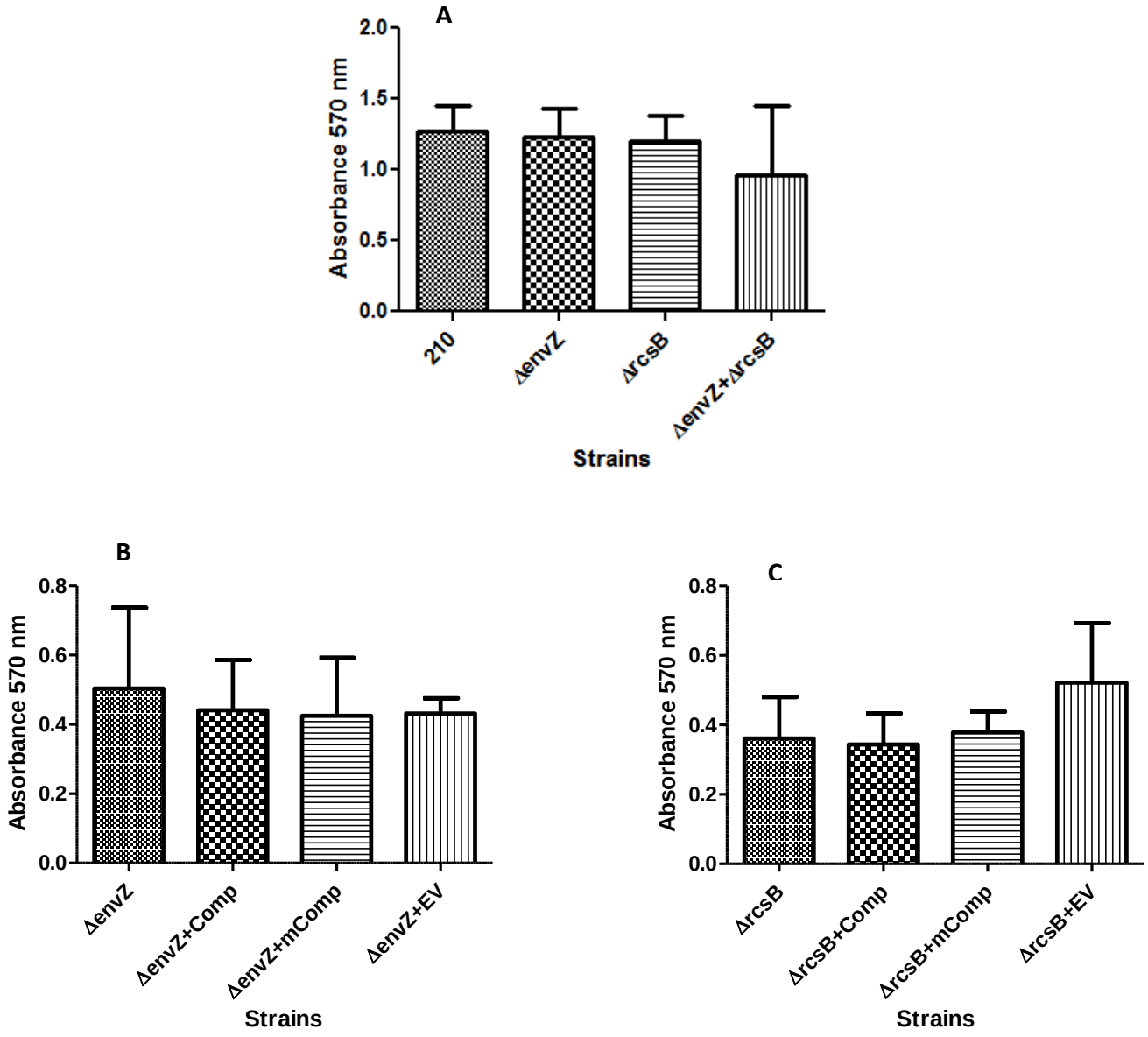


Figure 3. Biofilm Forming Capacity of Potential *S.* Typhimurium Hyper-biofilm Mutants
A| Biofilm production of several *S.* Typhimurium mutants lacking potential hyper-biofilm related genes. None of the deletions resulted in a significant change from wild-type biofilm production. Statistical analysis confirmed using a Student's T-test.
B| Biofilm production of *S.* Typhimurium *envZ* deletion mutants, including complemented wild type (+Comp), complemented mutant copy of *envZ* (+mComp), and an empty vector control (+EV). None of the mutants resulted in a significant change from the original deletion mutant biofilm production.
C| Biofilm production of *S.* Typhimurium *rcsB* deletion mutants, including complemented wild type (+Comp), complemented mutant copy of *rcsB* (+mComp), and an empty vector control (+EV). None of the mutants resulted in a significant change from the original deletion mutant biofilm production. No significant alterations in biofilm formation were observed among any of the mutants.

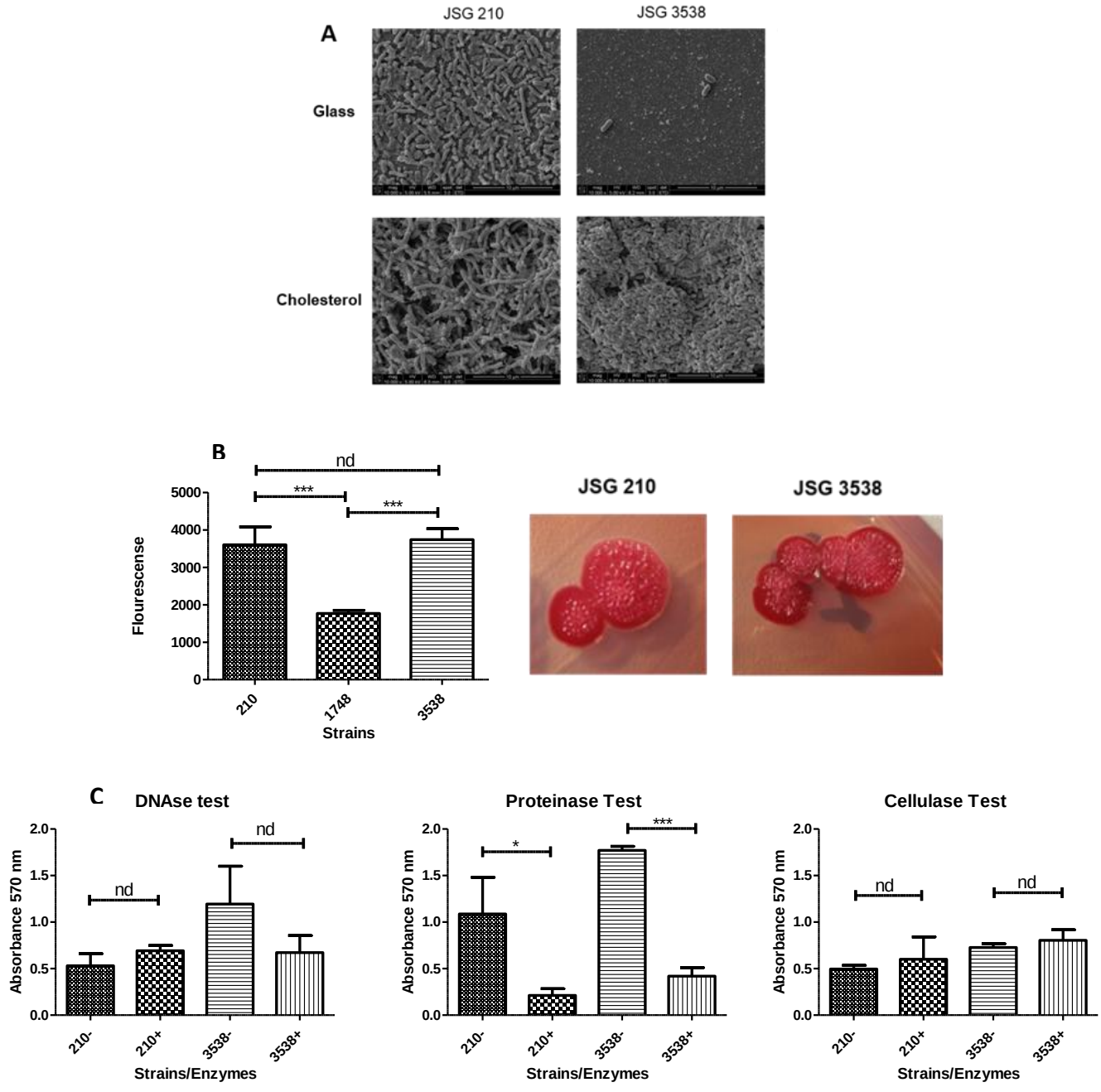
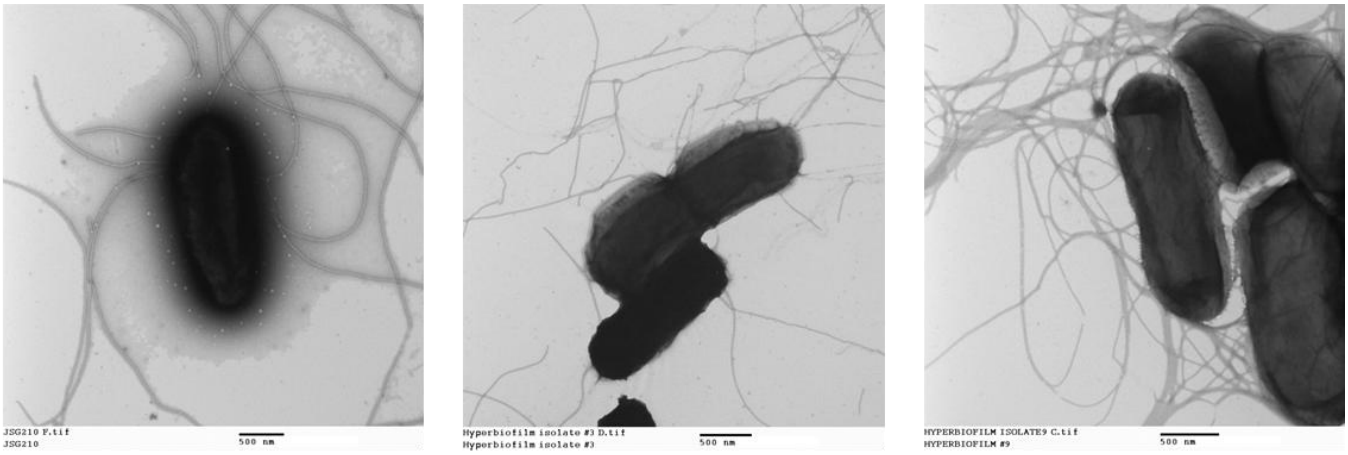


Figure 4 Biofilm Morphology and Determination of Composition
A| SEM pictures of JSG210 and JSG3538 biofilms on glass and cholesterol. JSG3538 grows a larger, more robust biofilm on cholesterol surfaces but is essentially absent from the glass surface.
B| Calcofluor assay comparing cellulose production between the wild type (JSG210) and the hyper-biofilm mutant (JSG3538). Both strains were also isolated on Congo Red plates and demonstrated the presence of cellulose and curli fimbriae.
C| Biofilm disruption assays comparing the effects of DNAse, proteinase, and cellulase on the biofilms of the wild type and hyper-biofilm strain. Both were very susceptible to the addition of proteinase and saw no effect from the addition of cellulase. DNAse appeared to have a greater effect on JSG3538, though the difference was not significant. Statistical analysis confirmed using a Student‘s T-test (*p<.05; ***p<.001)

A



Wild type | Hyperbiofilm Isolate 3 | Hyperbiofilm Isolate 9

B

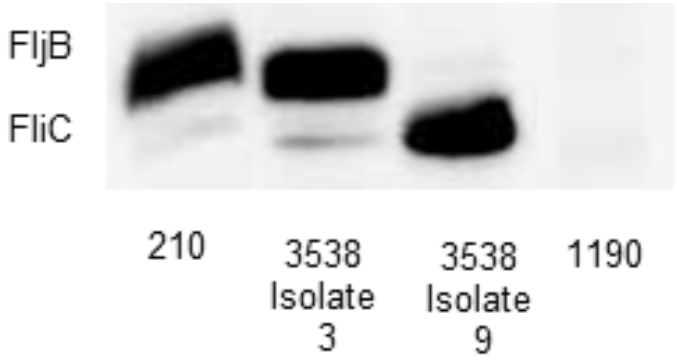


Figure 5. Evaluation of Cellular Appendages

A| TEM pictures of wild type *S.* Typhimurium and two hyper-biofilm isolates representing the relative low and high motility phenotypes respectively. Microscopy appears to show that the three isolates have drastically different cellular appendage phenotypes. These differing phenotypes suggest the expression of different types of appendages, though electron microscopy is unable to identify or quantify these forms.

B| Western blot comparing production of major flagellar subunits. Wild type *S.* Typhimurium (210) displays a $FljB^{+}/FliC^{+/-}$ genotype, the low-motility hyper-biofilm isolate (3538 isolate 3) displays a similar $FliB^{+}/FliC^{+/-}$ genotype, and the high-motility hyper-biofilm isolate (3538 isolate 9) displays a $FljB^{-}/FliC^{+}$ phenotype. All strains appear to display similar overall flagellar subunit production. A $FljB^{-}/FliC^{-}$ double knockout mutant (1190) was included as a negative control.

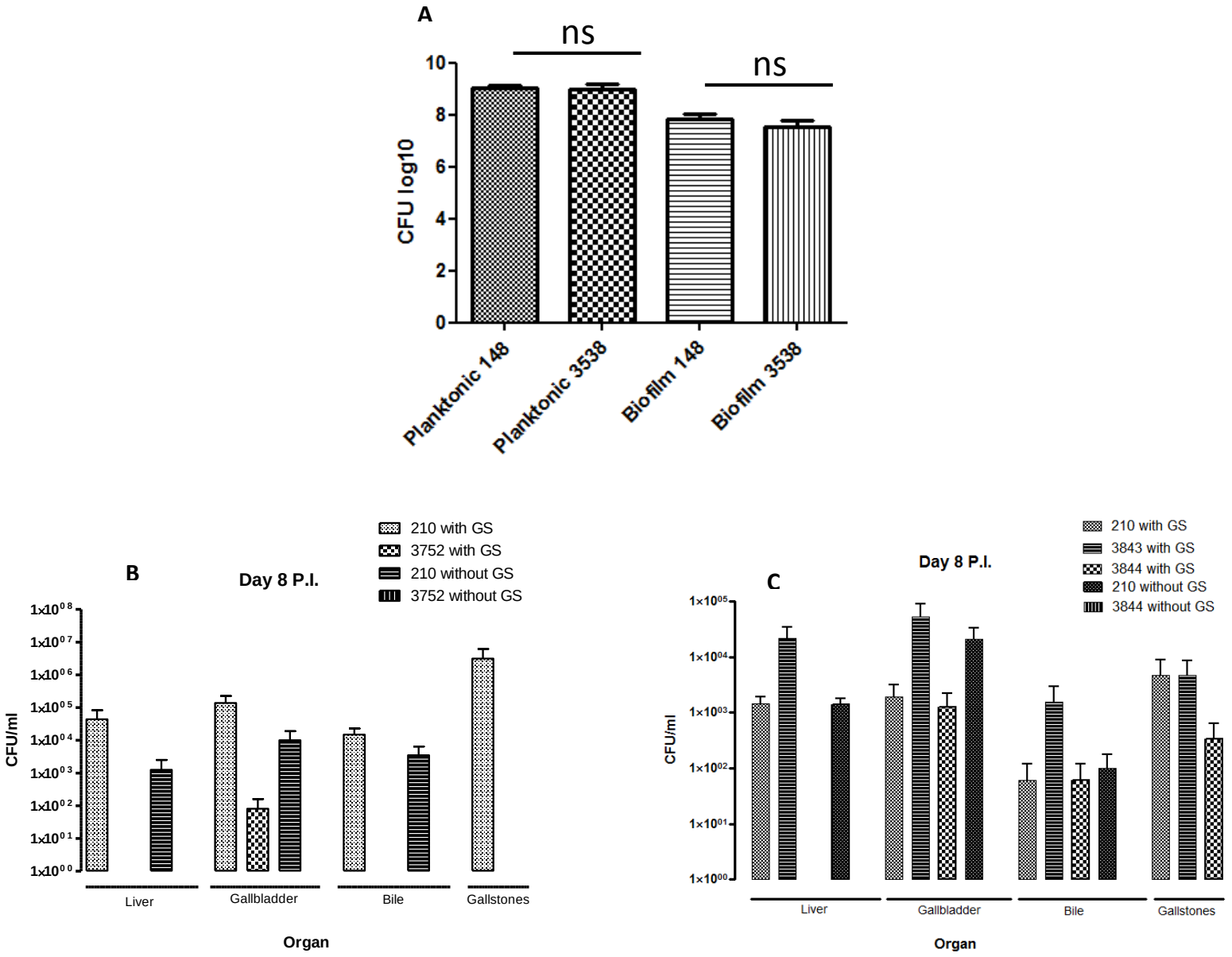


Figure 6. Virulence Properties

A| CFU enumeration following a biofilm competition assay between wild type *S.* Typhimurium and the hyper-biofilm strain. Colonization of the cholesterol surface was not significantly different between the two strains.

B| Mice were infected with wild type (210) or hyper-biofilm forming *S.* Typhimurium (3752). The hyper-biofilm strain demonstrated a severe systemic virulence defect with CFU recovered only from the gallbladder of a single mouse. This defect was present regardless of the gallstone status of the mouse.

C| For the second round of infections, two new strains were included, the wild type and hyper-biofilm strains complemented with envZ, a target for the cause of the systemic virulence defect, on a low-copy plasmid (3843 and 3844 respectively). Though the hyper-biofilm strain was still unable to colonize the liver, it more successfully invaded the gallbladder, although this increase had large variability between mice, indicating a lingering virulence defect unrelated to the truncation of envZ.

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